A Two-Amino Acid Change in the Hemagglutinin of the 1918 Influenza Virus Abolishes Transmission

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reduced but not abolished, were not as sensitive as *rtt109*Δ cells (Fig. 3, A and B). Moreover, *rtt109*Δ *H3-K56R* and *rtt109*Δ *H3-K56A* double-mutant cells displayed similar sensitivities toward these DNA-damaging agents as either single mutant alone (Fig. 3A and fig. S5). In contrast, cells expressing *rtt109* site-specific mutants where H3-K56 acetylation was not affected were resistant to these DNA-damaging agents (Fig. 3B and fig. S6). These results suggest that the ability of Rtt109 to suppress sensitivity toward DNA-damaging agents is mainly mediated by its HAT activity toward H3-K56.

In budding yeast, Rad52 forms spontaneous foci, predominantly during S and G_2-M phases of the cell cycle, and these foci are thought to be sites of repair of DNA lesions (*18*, *19*). Cells with mutations in proteins involved in DNA metabolism, such as Top3 exhibit elevated levels of Rad52 foci, possibly due to an increase in spontaneous chromosome breaks (*20*). The *rtt109*Δ and *H3-K56R* single- and double-mutant cells showed a substantial increase in Rad52 fused with yellow fluorescent protein (Rad52-YFP) foci (Fig. 3, C and D). Moreover, the *rtt109*Δ H3-K56R double-mutant cells did not exhibit more Rad52 foci than either *rtt109*Δ or *H3-K56R* mutant alone (Fig. 3D). Thus, the increase in Rad52-YFP foci observed in *rtt109*Δ mutant cells appears mainly to be due to loss of H3-K56 acetylation. Supporting this idea, acetylation of four other H3 lysine residues (K9, K14, K18, and K23) was not altered in the *rtt109*Δ mutant cells (fig. S7). Taken together, these data indicate that Rtt109-mediated acetylation of H3-K56 during S phase protects DNA from damage.

Here we have shown that Rtt109 is a member of a novel HAT family that acetylates H3-K56. The *rtt109*Δ mutant exhibited a synthetic lethal or slow-growth phenotype with a mutant allele of PCNA (proliferating cell nuclear antigen), *pol30-79*, which is defective in DNA replication and repair (*21*), but not with the PCNA mutant allele, *pol30-8,* which is defective in epigenetic silencing (*22*) (fig. S8A). The *rtt109*Δ mutant also exhibited a synthetic lethal/slow growth phenotype with a mutation in DNA polymerase α (fig. S8B) and was previously found to genetically interact with Orc2 and Cdc45 mutations (*23*, *24*). All of these proteins are involved in DNA replication. The genetic interactions between Rtt109 and the proteins involved in DNA replication suggest that the *rtt109*Δ mutant cells are defective in certain aspects of DNA replication. In support of this idea, the *rtt109*Δ mutant exhibits synthetic lethal or slow-growth phenotypes with mutations in genes such as *RAD52,* which are involved in homologous recombination (*25*), a process that is needed to resolve stalled replication forks (*26*). Thus, H3-K56 acetylation by Rtt109 is closely linked to DNA replication.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/315/5812/653/DC1
Materials and Methods
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A Two–Amino Acid Change in the Hemagglutinin of the 1918 Influenza Virus Abolishes Transmission

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The 1918 influenza pandemic was a catastrophic series of virus outbreaks that spread across the globe. Here, we show that only a modest change in the 1918 influenza hemagglutinin receptor binding site alters the transmissibility of this pandemic virus. Two amino acid mutations that cause a switch in receptor binding preference from the human α-2,6 to the avian α-2,3 sialic acid resulted in a virus incapable of respiratory droplet transmission between ferrets but that maintained its lethality and replication efficiency in the upper respiratory tract. Furthermore, poor transmission of a 1918 virus with dual α-2,6 and α-2,3 specificity suggests that a predominant human α-2,6 sialic acid binding preference is essential for optimal transmission of this pandemic virus. These findings confirm an essential role of hemagglutinin receptor specificity for the transmission of influenza viruses among mammals.

The "Spanish" influenza pandemic virus spread globally and resulted in the deaths of up to 50 million people worldwide (*1*, *2*). The ability of this H1N1 pandemic strain to spread rapidly and cause high rates of illness among humans makes it valuable for studying the molecular properties that confer efficient transmissibility of influenza viruses. An influenza virus bearing all eight gene segments of the 1918 pandemic virus was recently generated in cultured cells, was found to be lethal for chicken embryos and mice, and displayed a high-growth phenotype in human lung cells. Furthermore, the 1918 hemagglutinin (HA) and polymerase genes were shown to be essential for maximal virus replication and optimal virulence (*3*–*5*).

Influenza pandemics seem to occur every 10 to 40 years, but the factors that lead to the emergence of pandemic viruses are complex and poorly understood. However, the establishment of efficient and sustained human-to-human transmission of a virus to which humans have

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little or no preexisting immunity is a fundamental property of pandemic strains (*6*, *7*). Most threatening is the possibility of another pandemic, similar to that experienced in 1918, caused by a novel influenza subtype virus capable of causing severe respiratory disease and death. The avian influenza H5N1 virus, which has resulted in more than 250 human infections (*8*), has not acquired human influenza virus genes and lacks the ability to spread efficiently from human to human (*9*, *10*). Reassortment of avian H5N1 virus genes with human H3N2 influenza virus genes was shown to be insufficient for transmission of this avian virus (*11*), suggesting that additional unknown mutations are required for H5N1 to emerge as a pandemic strain.

The binding of influenza viruses to their target cells is mediated by the viral HA, which recognizes cell surface glycoconjugates containing terminal sialic acid (SA) residues. Avian influenza viruses preferentially bind SA linked to galactose by an α-2,3 linkage (α2,3 SA), which is found in high concentrations on the epithelial cells of the intestine of waterfowl and shorebirds (*12*). Conversely, human influenza viruses (H1 to H3 subtypes) more readily bind to receptors that contain terminal α-2,6-linked sialyl-galactosyl (α2,6 SA) moieties that are found on the human respiratory tract epithelium (*13*, *14*). The three influenza pandemic viruses of the last century, occurring in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2), each possessed an HA with a human α2,6 SA binding preference and are thought to have originated from an avian virus possessing the α2,3 SA binding preference (*13*–*16*). It has been postulated that the lack of sustained human-to-human transmission of avian influenza H5N1 viruses is due to their α2,3 SA receptor binding preference (*17*–*19*). Higher proportions of α2,3 SA receptors in the human lower respiratory tract compared with the upper respiratory tract may explain the severity of H5N1 viral pneumonia in humans resulting from H5N1 viral attachment deep in the lungs (*17*, *19*).

Amino acids at positions 190 and 225 in the 1918 pandemic influenza virus HA determine its receptor binding specificity (*15*, *16*). In this study, we generated recombinant influenza viruses possessing all eight gene segments of the 1918 influenza virus to examine the role of receptor binding specificity on replication, pathogenicity, and transmissibility of this pandemic strain. We generated two variant A/South Carolina/1/18 (SC18) 1918 viruses in which the HA was altered to change the receptor binding specificity from the parental human α2,6 SA (SC18) receptor preference to an avian α2,3 SA receptor preference (AV18) or a mixed α2,6 and α2,3 SA specificity reflecting the A/New York/1/18 (NY18) virus binding specificity. The NY18 virus was a natural variant sequenced from an archived lung tissue sample prepared during autopsy of a patient who died within 6 days of hospitalization in September 1918 (*20*). The HA corresponding to NY18 virus was made by introducing a single amino acid substitution [Asp^{225}→Gly^{225} (D225G)] in the SC18 HA. The AV18 virus, which differs by one amino acid from NY18 virus, was made by introducing an additional amino acid change [Asp^{190}→Glu^{190} (D190E)] within the NY18 HA. Compared with the SC18 virus, the AV18 variant has two amino acid changes (D190E and D225G) in the HA, which matches the conserved avian consensus sequence in the receptor binding site and which converts it to the classic α2,3 SA receptor preference (*15*). A/Duck/Alberta/35/76 (Dk/Alb) and A/Texas/36/91 (Tx/91) viruses were included in the study as controls representative of an avian

Table 1. Titer of virus stocks prepared on MDCK cells with trypsin (1 μg/ml, Sigma) and incubated at 37°C with 5% CO_2 for 48 hours. Hemagglutination assay of viruses used 0.5% α-2,3-resialylated CRBCs, α-2,6-resialylated CRBCs, or untreated CRBCs. The results shown correspond to four hemagglutination units. Similar results were obtained when viruses were adjusted to 8, 16, or 32 hemagglutination units with untreated CRBCs.

	Amino acid position (H3 numbering)			Presence or absence of hemagglutination		
	190	225	Infectivity titer (pfu/ml)	α2,6 CRBCs	α2,3 CRBCs	Untreated CRBCs
SC18	D	D	4.8×10^7	+	−	+
NY18	D	G	3.3×10^7	+	+	+
AV18	E	G	5.0×10^7	−	+	+
Dk/Alb	E	G	2.2×10^7	−	+	+

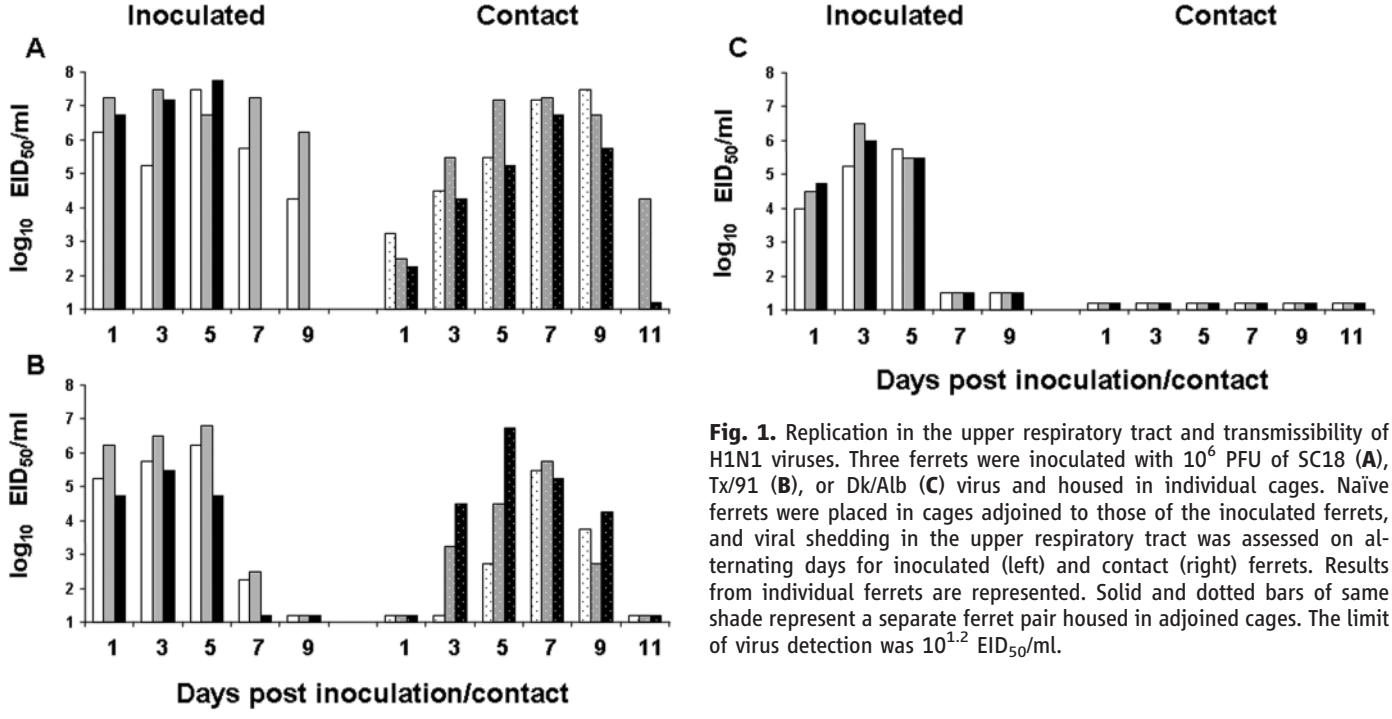

Fig. 1. Replication in the upper respiratory tract and transmissibility of H1N1 viruses. Three ferrets were inoculated with 10^6 PFU of SC18 (**A**), Tx/91 (**B**), or Dk/Alb (**C**) virus and housed in individual cages. Naïve ferrets were placed in cages adjoined to those of the inoculated ferrets, and viral shedding in the upper respiratory tract was assessed on alternating days for inoculated (left) and contact (right) ferrets. Results from individual ferrets are represented. Solid and dotted bars of same shade represent a separate ferret pair housed in adjoined cages. The limit of virus detection was $10^{1.2}$ EID_{50}/ml.

H1N1 virus and a human H1N1 virus, respectively. The 1918 viruses were generated by using the previously described reverse genetics system (*21*–*23*), and the identities of virus genes in the rescued viruses were confirmed by reverse transcription polymerase chain reaction and sequence analysis.

The rescued 1918 viruses containing the parental SC18 HA and the two variant HAs had similarly high infectivity titers in Madin-Darby canine kidney (MDCK) cells (Table 1). The receptor-binding properties of the 1918 viruses were confirmed in HA assays by using enzymatically modified chicken red blood cells (CRBCs) that contain either α2,3 or α2,6 SA, as previously described (*15*). The AV18 virus and the avian Dk/Alb control virus hemagglutinated the α2,3-resialylated CRBCs only, whereas the SC18 virus hemagglutinated the α2,6-resialylated CRBCs only. The NY18 virus hemagglutinated both α2,3- and α2,6-resialylated CRBCs.

Pathogenesis and transmissibility of the parental 1918 (SC18) virus were evaluated and compared with those of Tx/91 virus with an α2,6 SA receptor binding preference (*16*) and with those of the avian Dk/Alb virus possessing an α2,3 SA receptor binding preference (Table 1) (*24*). Ferrets were housed in adjacent cages that prevented direct and indirect contact between animals but allowed spread of influenza virus through the air (*11*, *25*). They were inoculated intranasally with 10^6 PFU (plaque forming units). One day after infection, three naïve ferrets housed in transmission cages were placed adjacent to each of the three inoculated ferrets (*26*). Three additional inoculated ferrets from each virus-infected group were killed on day 3 postinoculation (p.i.) for assessment of pathologic and virologic parameters (*26*). Ferrets inoculated with the parental SC18 virus shed high titers of infectious virus in nasal washes beginning as early as day 1 p.i. [50% egg infectious dose (EID_{50}/ml) from $10^{6.25}$ to $10^{7.25}$], and they sustained titers of $\geq 10^{4.5}$ EID_{50}/ml for 9 days p.i. (Fig. 1A, left). SC18 virus caused severe disease in all inoculated ferrets starting 2 days p.i.; symptoms included lethargy, anorexia, rhinorrhea, sneezing, severe weight loss (Table 2 and fig S1), and high fever, and two of the three animals died by day 11 p.i. Ferrets inoculated with H1N1 Tx/91 and Dk/Alb also shed high titers of virus in nasal washes (peak titers had EID_{50}/ml values from $10^{5.5}$ to $10^{6.8}$), but they were able to clear the virus from the upper respiratory tract by day 9 p.i. (Fig. 1, B and C) after displaying minimal symptoms (Table 2).

The human SC18 and Tx/91 viruses efficiently transmitted to each of the three contact ferrets (Fig. 1, A and B, right). The SC18 virus was detected in the contact ferrets as early as day 1 postcontact (p.c.), whereas the Tx/91 virus required 3 to 5 days to achieve detectable virus titers in nasal washes of the Tx/91 contact ferrets. The Tx/91 contact ferrets exhibited little morbidity, whereas all three SC18 contact ferrets exhibited severe signs of illness and weight loss, and one of three contact animals failed to clear the virus before it succumbed to infection on day 6 p.c. In contrast to the efficient spread of SC18 and Tx/91 viruses, the avian Dk/Alb virus was not transmitted to naïve contact ferrets, because virus was not detected in the nasal washes from the contact ferrets at any time. Furthermore, seroconversion was not detected by hemagglutination inhibition (HI) analysis of postexposure sera (Table 2). Both A/Duck/New York/15024/96 and A/Turkey/South Dakota/7034/86, which are representative avian viruses with an α2,3 SA receptor preference, exhibited efficient replication in the upper respiratory tract, but no transmission was detected between ferrets.

We introduced one– and two–amino acid substitutions into the 1918 virus HA to produce SC18 variants NY18 and AV18, respectively. A switch in receptor specificity from an α2,6 SA

Table 2. Clinical symptoms, virus replication, seroconversion, and transmissibility among ferrets inoculated with H1N1 viruses and among ferrets exposed to the inoculated animals (contacts). The percentage of mean maximum weight loss is shown. NW, nasal wash.

	Inoculated ferrets Number with characteristic/total number				Contact ferrets Number with characteristic/total number			Respiratory droplet transmission
	Sneezing (day of onset)	Weight loss (%)	Virus detected in NW	Seroconversion (range of HI antibody titer)	Weight loss (%)	Virus detected in NW	Seroconversion (range of HI antibody titer)	
SC18	3/3 (2)	3/3 (11.7)	3/3	1/1 (1280)*	2/3 (15.4)	3/3	3/3 (80–640)	Efficient
Tx/91	3/3 (2)	3/3 (6.2)	3/3	3/3 (160–640)	3/3 (3.5)	3/3	3/3 (160–320)	Efficient
Dk/Alb	2/3 (5)	2/3 (1.2)	3/3	3/3 (80–1280)	0/3	0/3	0/3	None
AV18	0/3	3/3 (14.7)	3/3	1/1 (640)*	0/3	0/3	0/3	None
NY18	0/3	3/3 (18.9)	3/3	2/2 (320–640)†	1/3 (1.4)	1/3	2/3 (40–80)	Inefficient

*Only one ferret survived and was tested. †Two ferrets survived and were tested.

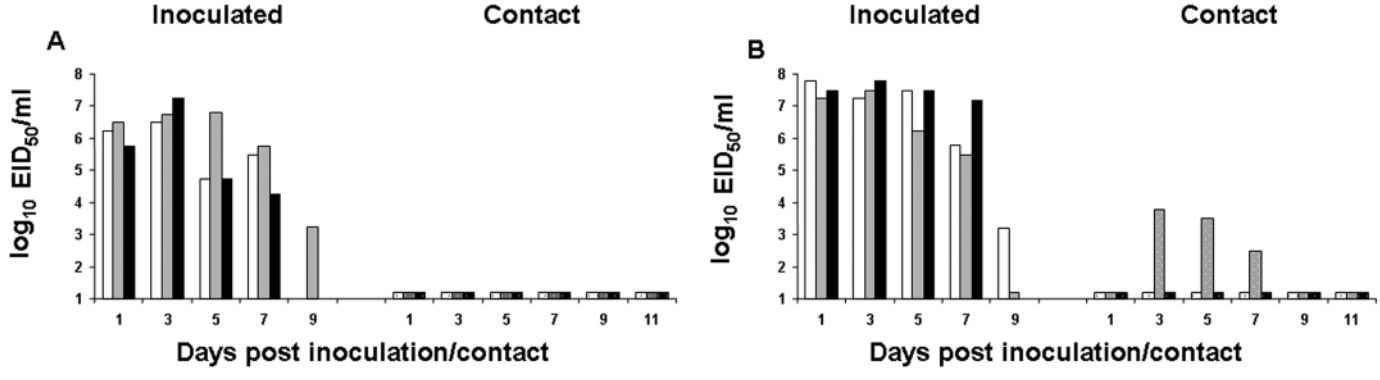

Fig. 2. Respiratory droplet transmissibility of 1918 viruses with mutated HA proteins. Three ferrets were inoculated with 10^6 PFU of AV18 (**A**) or NY18 (**B**) virus and placed in separate cages. Naïve ferrets were placed in cages adjoined to those of the inoculated ferrets, and viral shedding in the upper respiratory tract was assessed on alternating days for inoculated (left) and contact (right) ferrets. Results from individual ferrets are represented. Solid and dotted bars of same shade represent a separate ferret pair housed in adjoined cages.

(human) to an α2,3 SA (avian) binding preference abolished the transmissibility of the pandemic virus (Fig. 2 and Table 2). Although ferrets inoculated with AV18 virus exhibited severe illness (Table 2 and fig S1) and shed high titers of infectious virus in nasal washes (Fig. 2A, left), none of the three AV18 contact ferrets had detectable virus in nasal washes, and post-exposure sera collected from contact animals lacked antibodies against AV18. The NY18 virus, with dual α2,6 and α2,3 SA specificity, also resulted in severe illness and death among the inoculated ferrets, but it failed to transmit efficiently, as evidenced by the paucity of clinical symptoms and virus shedding among the contact ferrets (Fig. 2B). Two of the three NY18 contact ferrets seroconverted with relatively low HI titers of 40 and 80 (Table 2). The lack of efficient transmission was not due to the inability of the NY18 virus to replicate to high titers in the upper respiratory tract, including the nasal turbinates (Fig. 2B, left, and fig S2). Interestingly, no sneezing was noted among the AV18- and NY18-inoculated ferrets through a 14-day observation period, a finding consistent with the lack of notable sneezing observed in ferrets infected with H5N1 viruses (*11*).

Despite the differences in transmissibility of the parental 1918 (SC18) virus and the mutant 1918 viruses, similar damage to multiple lung lobes was observed 3 days after intranasal infection (*26*) (Fig. 3). Ferret lungs infected with SC18, AV18, and NY18 viruses exhibited necrotizing bronchiolitis and moderate to severe alveolitis with edema (Fig. 3, A to E, I, and J). Viral antigen was common in lung tissues, with localization in the upper to lower portions of the bronchial airways, bronchial and bronchiolar epithelium, and hyperplasic epithelium within alveoli (Fig. 3, F to H). Ferrets inoculated with control Tx/91 and Dk/Alb viruses generally showed a lack of significant lung lesions (Fig. 3, K to M).

Receptor binding, the initial event in influenza virus infection, was a major determinant of virus transmission efficiency of the H1N1 pandemic virus. This work also evaluates the virulence of the 1918 virus in a ferret model, a model that is believed to be more representative than the mouse model of disease caused by influenza viruses in humans. In contrast to other human influenza virus strains, the 1918 virus demonstrated uniquely high virulence and lethality in ferrets. The mutant 1918 virus possessing α2,3 SA receptor binding (AV18) was equally virulent in ferrets as the parental SC18 strain at the dose administered. Remarkably, the AV18 virus replicated in the upper respiratory tract as efficiently as the parental SC18 virus, but it failed to transmit to contact ferrets. Moreover, a human α2,6 SA binding preference is essential for optimal transmission of this exceptionally virulent virus. The introduction of a single mutation that converts the HA to dual α2,6 and α2,3 SA binding specificity (NY18) reduced the high transmissibility observed with the parental 1918 (SC18) virus. This result is consistent with the previously demonstrated lack of transmissibility of an H5N1 2003 virus that possessed dual α2,6 and α2,3 SA specificity due to a naturally acquired mutation at HA residue 223 (H5 numbering; residue 227 by H3 numbering) (*11*, *27*).

Our findings raise the possibility that, to become more transmissible, the currently circulating avian influenza H5N1 virus may require a receptor binding change to a predominant α2,6 SA binding preference. Such a modification of H5 HA may result in improved virus binding to

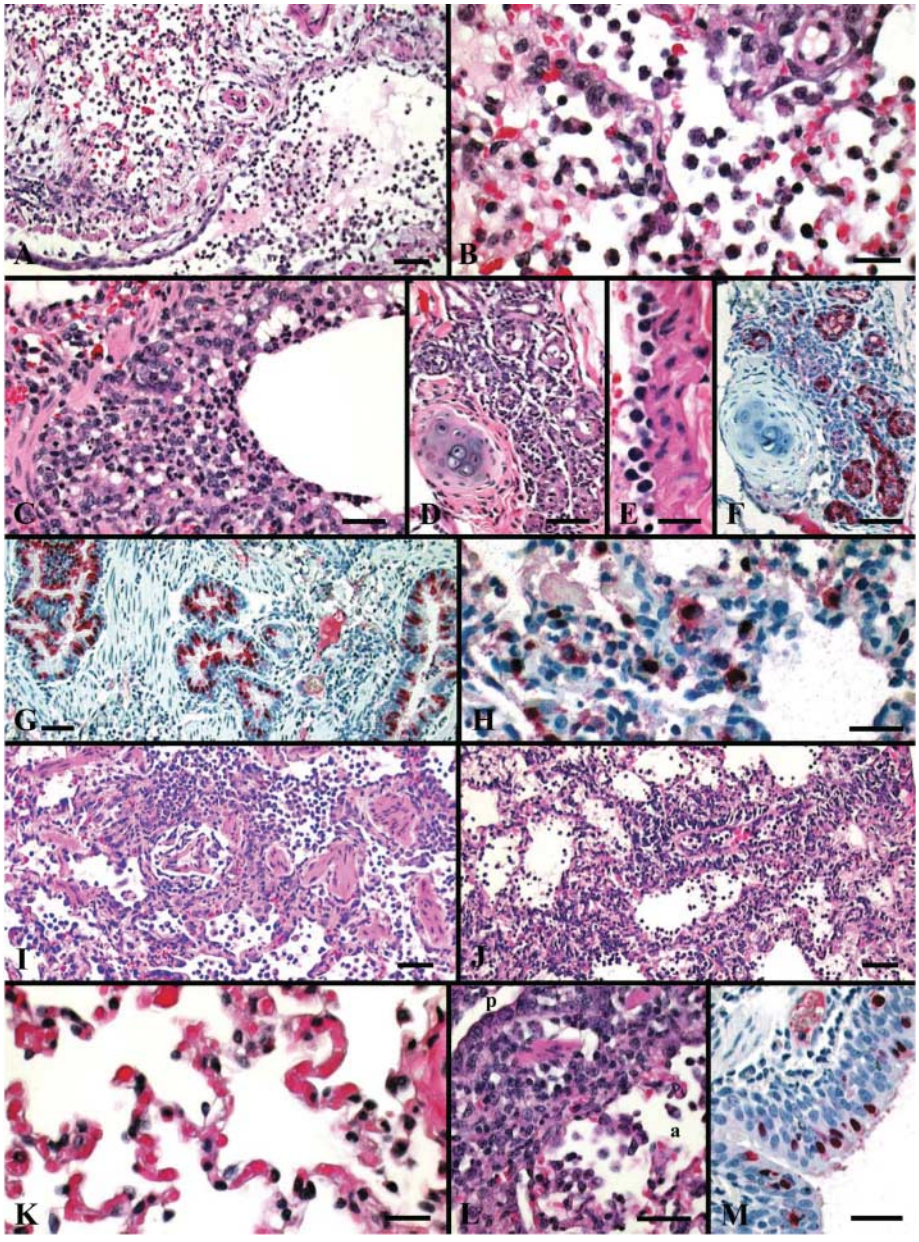

Fig. 3. Photomicrographs of hematoxylin and eosin [(A) to (E) and (I) to (L)] and immunohistochemically [(F) to (H) and (M)] stained lung sections from influenza virus–infected ferrets sampled on day 3 after inoculation. (**A** to **H**) Lung sections infected by SC18 virus. (A) Severe necrotizing bronchiolitis with severe diffuse alveolitis and edema. Scale bar indicates 50 μm. (B) Severe diffuse alveolitis; scale bar, 20 μm. (C) Necrotizing bronchiolitis; scale bar, 30 μm. (D) Necrosis and (F) associated influenza viral antigen in submucosal serous glandular epithelium of a bronchus; scale bar, 50 μm. (E) Margination and adhesion of neutrophils to endothelial cells of a pulmonary arteriole; scale bar, 20 μm. (G) Influenza viral antigen in epithelium of a primary bronchiole; scale bar, 50 μm. (H) Viral antigen commonly in macrophages and alveolar epithelial cells; scale bar, 20 μm. (**I**) NY18 virus; severe diffuse alveolitis with accompanying necrotizing bronchiolitis; scale bar, 50 μm. (**J**) AV18 virus; diffuse severe alveolitis and edema with necrotizing bronchiolitis; scale bar, 50 μm. (**K**) Tx/91 virus; normal alveoli; scale bar, 15 μm. (**L**) Dk/Alb virus, purulent bronchiolitis (p) with peribronchiolar mixed cell inflammation and associated moderate alveolitis (a); scale bar, 50 μm. (**M**) Dk/Alb viral antigen in bronchial epithelium; scale bar, 30 μm.

human tracheal epithelial cells expressing high amounts of terminal α2,6 SA motifs and, simultaneously, in an improved ability to overcome the inhibitory effects of human bronchial mucins associated with α2,3 SA receptors (*28*). However, mutations that caused a shift from the avian-type to human-type receptor binding specificity for the H1 subtype do not cause an equivalent shift in specificity for the H5 subtype (*24*). Likewise, the amino acid changes required to alter the H3 HA from an avian- to human-type receptor binding specificity are different from those required for the H1 HA. Therefore, it is likely that different avian HA subtypes have different structural requirements to confer receptor specificity. Thus, it is currently unknown which additional mutations in the H5 HA would cause a shift to the human-type specificity, which may be required for H5N1 viruses to transmit efficiently among humans.

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Protein Kinase C β and Prolyl Isomerase 1 Regulate Mitochondrial Effects of the Life-Span Determinant p66^{Shc}

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The 66-kilodalton isoform of the growth factor adapter Shc (p66^{Shc}) translates oxidative damage into cell death by acting as reactive oxygen species (ROS) producer within mitochondria. However, the signaling link between cellular stress and mitochondrial proapoptotic activity of p66^{Shc} was not known. We demonstrate that protein kinase C β, activated by oxidative conditions in the cell, induces phosphorylation of p66^{Shc} and triggers mitochondrial accumulation of the protein after it is recognized by the prolyl isomerase Pin1. Once imported, p66^{Shc} causes alterations of mitochondrial Ca^{2+} responses and three-dimensional structure, thus inducing apoptosis. These data identify a signaling route that activates an apoptotic inducer shortening the life span and could be a potential target of pharmacological approaches to inhibit aging.

The protein p66^{Shc} (*1–4*) is an alternatively spliced isoform of a growth factor adapter that is phosphorylated upon oxidative stress (*2*). Ablation of the p66^{Shc} gene causes life-span prolongation with no pathological consequence (*2*). A fraction of p66^{Shc} localizes to mitochondria (*3–5*), where it binds to cytochrome c and acts as oxidoreductase, generating reactive oxygen species (ROS) and leading to organelle dysfunction and cell death (*5*). The route leading to p66^{Shc} activation is still unclear. Phosphorylation of a critical serine (Ser^{36}) is necessary (*2*), but the kinase responsible has not been identified. Moreover, mitochondrial p66^{Shc} is unphosphorylated, indicating that additional regulatory elements must exist.

Mitochondria receive, under stimulation by physiological agonists or toxic agents, Ca^{2+}-mediated inputs (*6–8*) that are decoded into effects as diverse as metabolic stimulation and apoptosis (*9*). Ca^{2+} responsiveness is a highly sensitive readout of mitochondrial state: Partial defects in mitochondrial energization, as in mitochondrial diseases, cause defects in Ca^{2+} handling by the organelle (*10*). Moreover, mitochondrial Ca^{2+} uptake is modulated by regulatory proteins such as kinases. Some protein kinase C (PKC) isoforms (*11*) specifically affect the responses of mitochondrial Ca^{2+} to agonists (PKCβ reduces them, whereas PKCζ enhances them) (*12*). PKCs are also proposed to be activated in conditions of oxidative stress (*13*). We therefore used aequorin to monitor cellular concentrations of Ca^{2+}, a green fluorescent protein with mitochondrial presequence (mtGFP) to monitor organelle structure, and other molecular tools to clarify the signaling route linking the oxidative challenge to the activation of p66^{Shc} proapoptotic effect within

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